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The Genetically Remote Pathogenic Strain NVH391-98 of the *Bacillus cereus* Group Is Representative of a Cluster of Thermophilic Strains▽†

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Bacteria of the *Bacillus cereus* group are known to cause food poisoning. A rare phylogenetically remote strain, NVH391-98, was recently characterized to encode a particularly efficient cytotoxin K presumably responsible for food poisoning. This pathogenic strain and its close relatives can be phenotypically distinguished from other strains of the *B. cereus* group by the inability to grow at temperatures below 17°C and by the ability to grow at temperatures from 48 to 53°C. A temperate phage, phBC391A2, residing in the genome of NVH391-98 allows us to distinguish the three known members of this thermophilic strain cluster.

The *Bacillus cereus* group includes gram-positive aerobic spore-forming bacteria commonly found in soil and sometimes implicated in food poisoning. These opportunistic pathogens cause gastrointestinal diseases manifested by diarrheic or emetic syndromes (5, 12). The practical importance of *B. cereus* group studies is growing because of the increasing number of related food poisoning cases, especially in developed countries. Since this problem has an obvious relevance to the ability of some *B. cereus* strains to multiply in chilled products, psychrotolerant strains have been the focus (3, 4, 8, 22). It was shown that psychrotolerant and mesophilic strains have optimal growth temperatures in the range of 25 to 35°C, but psychrotolerant strains can be distinguished by their ability to grow at 4 to 7°C but not at 43°C (14). Based on several distinctive features of psychrotolerant strains, including the presence of a specific signature in the 16S rRNA sequences, a new species, *Bacillus weihenstephanensis*, was proposed (14, 21). In contrast, the data available in regard to the ability of the *B. cereus* group bacteria to grow at moderately high temperatures, that is, close or slightly higher than 50°C, are scarce, nonsystematic, and not sufficiently detailed. Some isolates were reported to grow on plates at 55°C after 5 days of incubation (18). A strain, NVH200, was able to grow at up to 50°C in a liquid medium after a long lag phase of 72 h (1). Here we show that only a very few strains of the *B. cereus* group are able to grow at temperatures higher than 48°C. In fact, this ability seems to be restricted to a few strains represented by the genetically remote strain NVH391-98, isolated from a severe food poisoning outbreak, which caused three fatal cases (15). This strain is able to synthesize in elevated amounts a particularly efficient diarrheic cytotoxin K (2, 7). NVH391-98 and its close relatives are the unique thermophilic isolates of the *B. cereus* group, presumably representing another novel species.

NVH391-98 represents a cluster of thermophilic strains. Psychrotolerant *B. weihenstephanensis* strains KBAB4 (20, 24) and WSBC10206 (14), obtained from V. Sanchis (INRA, La Minière, France) and S. Scherer (IM, Freising, Germany), were isolated from soil in France and Germany, respectively. Mesophilic *B. cereus* strains ATCC 14579 (9), obtained under the designation 6A5 from D. R. Zeigler (BGSC, Columbus, OH), and ATCC 10987 (19), obtained from D. Lereclus (INRA, La Minière, France), were of air and dairy origin from the United Kingdom and Canada, respectively. The strain NVH391-98, obtained from D. Lereclus, was isolated from a vegetable purée in France in 1998 (15). The strains INRA AF2, obtained under the designation INRA398, and NVH883/00 (10) were from M.-H. Guinebretiere (INRA, Avignon, France). *Bacillus subtilis* 168 was from the laboratory collection. Standard manipulations with bacteria, phages, and DNA were done as described previously (16).

FIG. 1. Growth of different *Bacillus* strains at 30°C and 48°C. *B. subtilis* 168 is used as a positive control for high-temperature growth. The strains *B. cereus* ATCC 14579, *B. weihenstephanensis* KBAB4, and NVH391-98 are of the *B. cereus* group.

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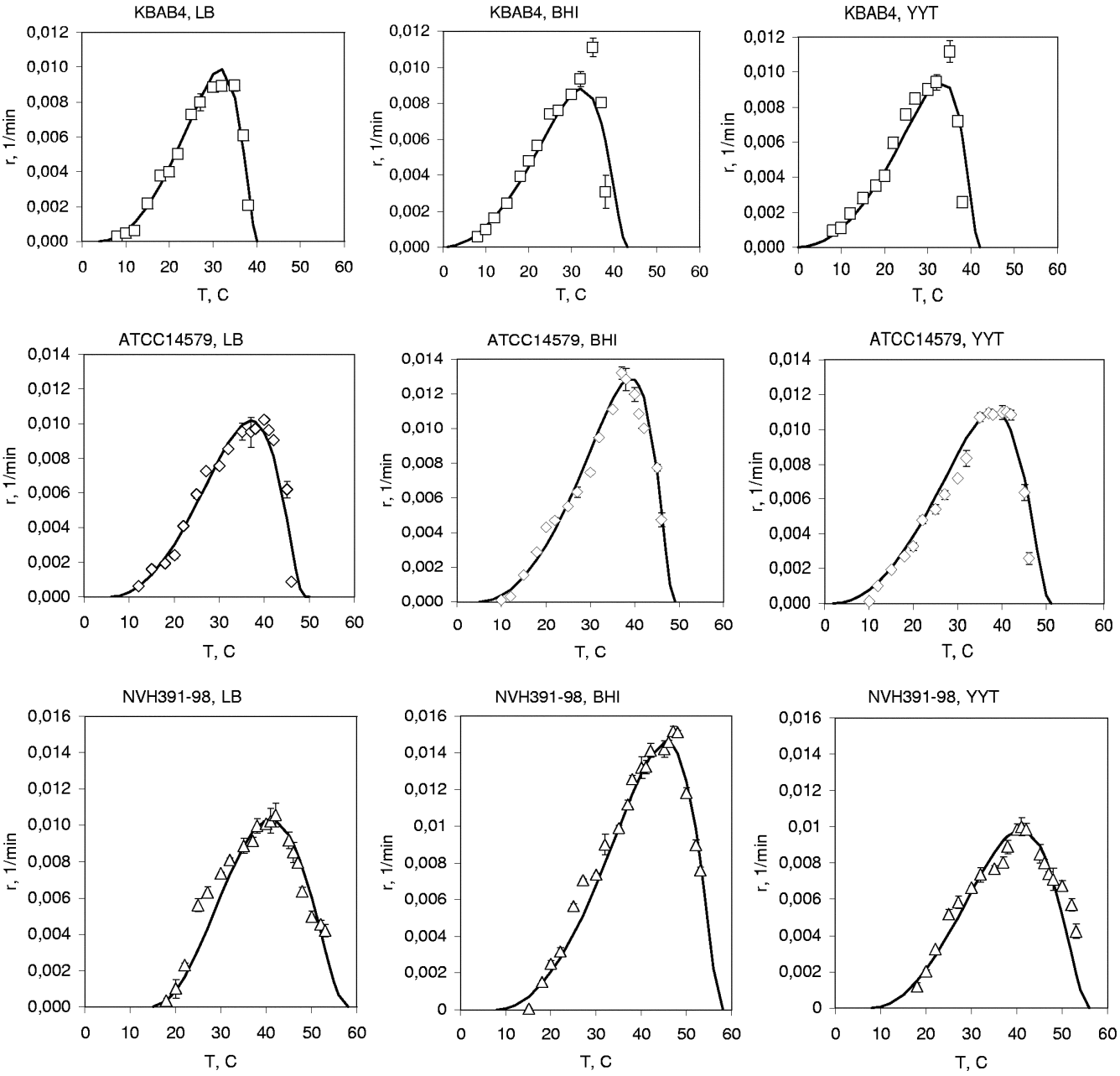

FIG. 2. Growth of *Bacillus cereus* group strains at different temperatures. Growth rates (r) for representatives of psychrotolerant (*B. weihenstephanensis* KBAB4), mesophilic (*B. cereus* ATCC 14579), and thermophilic ("*B. cytotoxicus*" NVH391-98) strains in different media are plotted against the growth temperature (T). The experimental points are the mean values of three independent experiments. The solid lines show nonlinear least-squares approximations according to the Ratkowsky equation (17). C, degrees Celsius.

In a preliminary experiment, we noted that strain NVH391-98 was able to grow rapidly at 48°C, while none of the other tested strains of the *B. cereus* group grew at this temperature. This is illustrated by a simple plate growth test (Fig. 1). The *B. subtilis* strain 168, known to be able to grow up to 52°C (11), was used as the high-temperature growth control. This experiment clearly indicated that at least at the high temperature, there is a large difference in the growth abilities of strain NVH391-98 and other representatives of the *B. cereus* group. To characterize these differences quantitatively, we examined the growth of five strains of the *B. cereus* group, including two psychrotolerant strains, two mesophiles, and NVH391-98. We used three different liquid media, Luria-Bertani (LB), brain heart infusion, and LB medium supplemented with 6 g/liter Bacto tryptone and 5 g/liter yeast extract, at temperatures ranging from 8 to 55°C.

For each strain, an aliquot of the overnight culture was diluted 100-fold into fresh medium. Growth was monitored by measuring the increase of the optical density at 600 nm with an automatic cell growth analyzer (Bioscreen C; Labsystems). Mi-

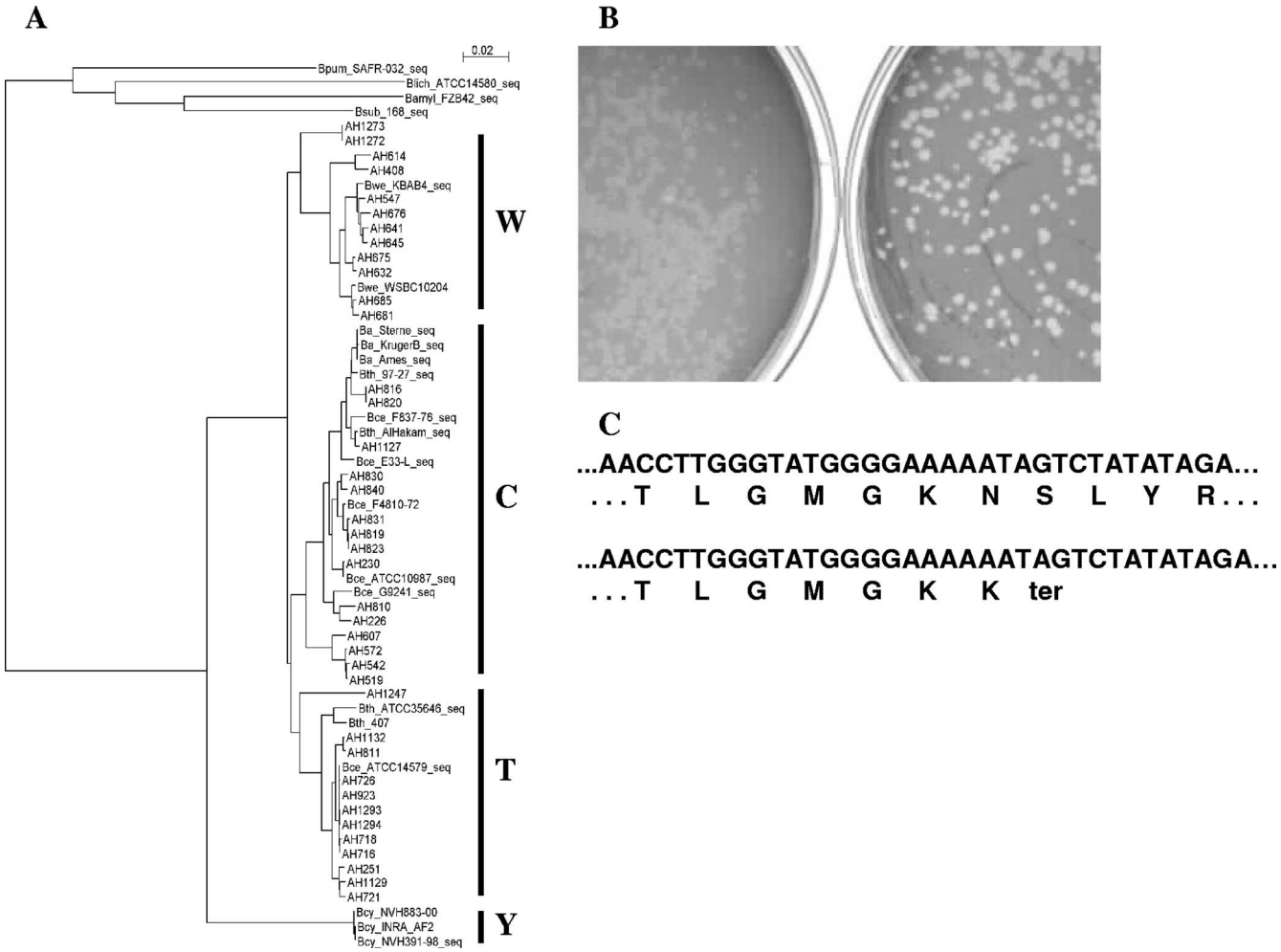

FIG. 3. Phylogenetic and phenotypic distinctions of the "*Bacillus cytotoxicus*" strain cluster. (A) Neighbor-joining phylogenetic tree for a representative set of the *B. cereus* group strains. Strain names and concatenated sequences are taken from the MLST database described in reference 23, GenBank, or genomic sequences. Vertical bars labeled C, T, and W indicate the major strain clusters according to reference 20. The cluster of the three strains closest to NVH391-98 is labeled Y. The sequences of the four bacillus strains closest to the *B. cereus* clade, *B. subtilis* 168, *Bacillus amyloliquefaciens* FZB42, *Bacillus licheniformis* ATCC 14580, and *Bacillus pumilis* SAFR-032, were extracted from GenBank entries (accession no. NC_000964, NC_009725, NC_006270, and NC_009848) and used to represent an out-group. Completely sequenced strains are labeled seq. (B) Turbid (left) and clear (right) plaques formed by phage phBC391A2 and by its mutant, phBC391B3vir, respectively, on the INRA AF2 strain used as an indicator strain. (C) Partial nucleotide and corresponding amino acid sequences of the Bcer982969 gene in the phages phBC391A2 (top) and phBC391B3vir (bottom). The location of this mutation in the putative repressor gene of phBC391A2 proves the identity of phBC391B3vir as a clear-plaque mutant of the former phage.

crotiter plates containing 300 μl per well were shaken continuously for 96 h.

Extrapolation of the growth profile curves for the psychrotolerant *B. weihenstephanensis* strains KBAB4 and WSBC 10206 to the zero growth rates, using a model of Ratkowsky et al. (17), indicated a minimal theoretical growth temperature of 1 to 4°C. Experimentally, growth was detected at the minimal temperature of 8°C (Fig. 2) (see the data for all tested strains and the equation parameters in the supplemental material). Above the optimal growth temperature of 30 to 32°C, the specific growth rates rapidly decreased with an increase of temperature. Above 38°C, no growth was experimentally detected, while the theoretical maximal growth temperature was in the range of 40 to 46°C. For the two mesophilic strains, the experimentally detected minimal growth temperature was 12°C, while 2 to 7°C was the theoretical estimation, and an optimal growth was observed between 35 and 40°C (Fig. 2) (see the supplemental material for all data). The *B. cereus* strain ATCC 10987 was able to grow at up to 47°C, while the *B. cereus* strain ATCC 14579 did not grow above 46°C. The theoretical maximal growth temperature for the mesophilic strains was in the range of 49 to 53°C, again slightly higher (1°C) for the *B. cereus* strain ATCC 10987. Multiple plate tests for about 100 *B. cereus* group strains from our laboratory collection (not shown) confirmed the general conclusion that almost all strains of the *B. cereus* group are able to maintain experimentally detectable growth between 8 and 47°C but not beyond this range. If the Ratkowsky model adequately describes the whole range of the temperature dependence of growth, these temperatures can be extended to 2 to 53°C. Presumably, longer incubation times

are needed to detect the slow growth at the extreme temperatures, but also some growth induction constraints may exist in our experimental conditions that do not allow us to detect growth up to the theoretical limits. These constraints can result in very long lag times, hampering the measurements of very slow exponential-phase growth. That is why it is necessary to apply a theoretical model for the estimation of the extreme growth temperatures. The strain NVH391-98 did not grow at temperatures below 18°C but was able to grow at up to 53°C (Fig. 2). The optimal growth temperature was 40°C in LB medium, 42°C in LB medium supplemented with 6 g/liter Bacto tryptone and 5 g/liter yeast extract, and 46°C in brain heart infusion medium, displaying a relatively high dependence of growth ability of the strain on the medium used. The theoretical limits of growth, based on Ratkowsky's model, were 8 to 15°C and 58°C. The strain NVH391-98 is therefore able to grow at temperatures 6 to 8 degrees higher than the mesophilic strains of the *B. cereus* group.

Phylogenetic remoteness of NVH391-98 and distinction from its close relatives. The phylogenetic remoteness of the strain NVH391-98 from other representatives of the *B. cereus* group was demonstrated recently (6, 13). Figure 3A illustrates this using the multiple locus sequence typing (MLST) schema proposed by Tourasse et al. (23) for the extended set of strains in comparison with that reported earlier (6). This comparison includes the sequences of the strains NVH883/00 (GenBank accession no. EF108377 to -383) and INRA AF2, which are closely related to NVH391-98 (6, 10) and have similar thermophilic phenotypes. We resequenced the relevant loci for NVH883/00 and confirmed the differences with NVH391-98. MLST and several other independently determined sequences (accession no. EF108376 and EF108384 to -390) for the strain INRA AF2 do not allow us to distinguish it from NVH391-98.

We noted that after overnight propagation on solid media at 50°C and subsequent longtime storage at room temperature, two strains, NVH391-98 and INRA AF2 (but not NVH883/00), show typical autolytic morphology, characterized by plaque-like clearings in the areas of dense growth on agar. Using double-layer agar assays, we found that the NVH391-98 strain produces bacteriophage that formed turbid plaques on the INRA AF2, but not the NVH883/00, cell lawn. This phenotype allows us to distinguish the NVH391-98, INRA AF2, and NVH883/00 strains. We detected clear-plaque-forming phage mutants among turbid plaques on INRA AF2 with a frequency of approximately 2×10^{-4} (Fig. 3). Analysis of the genomic sequence of the strain NVH391-98 (accession no. NC009674) revealed two regions, nearly 2,690 and 3,010 kb, designated phBC391A1 and phBC391A2, respectively, containing clusters of phage-related genes and thus potentially encoding inducible prophages.

To identify the phage infecting the strain INRA AF2, we isolated DNA from a clear-plaque-forming phage mutant, designated phBC391B3vir (Fig. 3B), and analyzed it by EcoRV digestion. The resulting profile corresponded to the theoretical profile of phage phBC391A2 DNA, rather than to that of phBC391A1 (not shown). Direct sequencing of this DNA by use of primers specific to NVH391-98 chromosomal DNA produced readable chromatograms, with the average signal strength 7- to 10-fold higher, only with primers corresponding to the phBC391A2 (not shown). Moreover, we determined that the mutation causing the clear-plaque phenotype of phBC391B3vir was due to the insertion of an additional A into the stretch of five A's, resulting in a reading frame shift in the Bcer982969 gene encoding a potential phage repressor (Fig. 3C). Therefore, the three strains can be phenotypically distinguished by using their different levels of susceptibility to the phage phBC391A2. Only the strain NVH391-98 produces this phage, and only the strain INRA AF2 can be used as an indicator strain to detect PFU.

In conclusion, the formal sequence-based comparisons allow us to consider the strain NVH391-98 and its close relatives as a genetically rather remote species of the *B. cereus* group. At present, NVH391-98 and its two close relatives described here are the only known strains of this group for which thermophilic growth is confirmed. We proposed earlier that strain NVH391-98 be considered a representative of a new species, for which the name "*Bacillus cytotoxicus*" was suggested (13). The existence of two closely related but different strains, INRA AF2 and NVH883/00, validates the novel species status of NVH391-98.

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